Differential Protection Against Papaya Ringspot Virus Isolates in Coat Protein Gene Transgenic Papaya and Classically Cross-Protected Papaya

P. F. Tennant, C. Gonsalves, K.-S. Ling, M. Fitch, R. Manshardt, J. L. Slightom, and D. Gonsalves

First, second, and seventh authors: Department of Plant Pathology, Cornell University, Geneva, NY 14456; fourth author: U.S. Department of Agriculture, ARS P.O. Box 1057, Aeia, HI 96701; fifth author: Department of Horticulture, University of Hawaii, Honolulu, HI 96822; and sixth author: Molecular Biology Research Unit 7242, The Upjohn Company, Kalamazoo, MI 49001.

Partially supported by grants from the Cornell Biotechnology Program sponsored by the New York State Science and Technology Foundation, a consortium of Industry and the National Science Foundation, and by the U.S. Department of Agriculture under CSRS Special Grant 88-34135-3607, managed by the Pacific Basin Advisory Group (PBAG).

We thank the Jamaica Agricultural Development Foundation (JADF) for supporting P. F. Tennant and S. Ecker-Day and D. Hummer for their technical assistance.

Accepted for publication 11 August 1994.

ABSTRACT

Tennant, P. F., Gonsalves, C., Ling, K.-S., Fitch, M., Manshardt, R., Slightom, J. L., and Gonsalves, D. 1994. Differential protection against papaya ringspot virus isolates in coat protein gene transgenic papaya and classically cross-protected papaya. Phytopathology 84:1359-1366.

Transgenic papaya expressing the coat protein gene of the mild papaya ringspot virus strain from Hawaii (PRV HA 5-1) showed high levels of resistance against the severe PRV HA isolate from Hawaii. Inoculation with high concentrations of the virus, multiple mechanical inoculations, or graft inoculations failed to break the resistance of transgenic papaya. Virus recovery assays from these inoculated plants suggested that virus replication and movement were impaired. Transgenic papaya also showed high levels of resistance against severe PRV isolates recently collected from Hawaii. Similarly, PRV HA 5-1 cross-protected papaya offered high levels of protection against two of the three isolates from Hawaii. However, neither transgenic nor mild strain-infected papaya showed good levels of protection against PRV isolates from 11 other geographical regions that were serologically related to PRV HA 5-1. A range of reactions was observed: complete resistance; delay in symptom development and symptom attenuation with PRV isolates from the Bahamas, Florida, and Mexico; and, a shorter delay in symptom development but no symptom attenuation with isolates from Brazil or Thailand.

Papaya ringspot virus (PRV) causes one of the most important diseases in papaya (*Carica papaya* L.) and occurs wherever papaya are grown (20). PRV is a potyvirus and is nonpersistently transmitted by aphids to papaya and members of the Chenopodiaceae and Cucurbitaceae families (20). The PRV strains that infect papaya are designated PRV-p and are differentiated from the PRV-w strains (formerly watermelon mosaic virus I) that are economically important viruses of cucurbits. Papaya trees infected with PRV-p are stunted, produce disfigured fruits with ringspots, and have decreased yields (20).

Efforts to control PRV on papaya have had limited success. Control by conventional breeding with the incorporation of PRV-resistant genes of wild *Carica* species into the commercial varieties is difficult due to interspecific reproductive barriers (14). Tolerant varieties are available, but their generally poor fruit quality and partial loss of tolerance when backcrossed to susceptible germ plasm limit their usefulness. The disease is, however, controlled in some areas of Hawaii by cross protection (15). Cross protection is a natural form of pathogen-derived resistance (23) and involves the use of a mild virus strain to protect plants against economic damage caused by challenge inoculation of a severe strain of the same virus or a related virus (8). The mild PRV strain (PRV HA 5-1) used in Hawaii was derived from a severe Hawaiian PRV strain (PRV HA) (29). Although cross protection has been successfully used in Hawaii, it has had limited success in controlling the disease elsewhere. In Taiwan, cross protection allows for the production of marketable fruits in isolated regions of low disease pressure (27) and there is no protection against isolates from Thailand or Mexico (29).

Given the reports that coat protein-mediated protection (CPMP), which is a form of pathogen-derived resistance (23), has considerable potential in controlling plant virus diseases (1), the coat protein (CP) gene of the mild PRV HA 5-1 strain was cloned (21) and used to transform Hawaiian papaya cultivars (6,7). Under greenhouse conditions, an R_0 transgenic line of the cultivar Sunset, designated 55-1, was highly resistant against mechanical inoculation with the closely related severe Hawaiian PRV HA (7). The plants remained symptomless and virus was not recovered by back inoculation to papaya, *Cucumis metuliferus*, or the local lesion host *Chenopodium quinoa*. A field trial with clones of the R_0 55-1 transgenic line was initiated in April 1992. After 24 mo, the transgenic papaya are still symptomless (R. Manshardt, *unpublished*).

Since papaya are almost always propagated by seed, and generation cycles are a year or less, this resistant transgenic papaya line 55-1 could serve as a germ plasm source for a breeding program aimed at the control of PRV on a worldwide basis. However, success of this germ plasm would depend on its resistance to PRV isolates from different geographical regions where papaya is grown. Previous data suggested that this transgenic papaya might provide protection against various isolates of PRV because transgenic tobacco, a nonhost of PRV, expressing the same CP gene of PRV HA 5-1, was protected against detrimental effects of three potyviruses: tobacco etch virus, potato virus Y, and pepper mottle virus (13). On the other hand, infection with the mild PRV HA 5-1, which provided the CP gene of the line 55-1, does not afford protection against PRV isolates from Thailand and only limited protection against those from Taiwan (27,29).

Thus, it is necessary to test the resistance of the transgenic papaya against PRV isolates from diverse geographical locations. In this report data are presented on the reactions of progenies of the transgenic line 55-1 and classically cross-protected papaya against PRV isolates from Hawaii and diverse geographical regions. It is concluded that both CPMP and classical cross protection provide high levels of protection against PRV isolates

from Hawaii but neither practice provides broad protection against PRV isolates from different geographical regions.

MATERIALS AND METHODS

Transgenic papaya. Greenhouse grown transgenic R_0 female Sunset 55-1 papaya (6,7) were pollinated in Geneva, NY, with pollen of nontransgenic Sunrise papaya obtained from Hawaii. The resulting seeds were used to produce seedlings for subsequent tests in Geneva.

Detection of NPTII, GUS, and PRV CP gene expression in transgenic papaya. Neomycin phosphotransferase II (NPTII) expression (3) was detected in papaya leaf disks (2 mg) homogenized (1:20, w/v) in extraction buffer (0.25 M potassium phosphate, 0.1 M EDTA, pH 7.5) by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's conditions (5 Prime-3 Prime Inc., Boulder, CO). The absorbance was measured at 405 nm with a MicroELISA AutoReader (Dynatech Inc., Chantilly, VA) 10 min after the addition of *p*-nitrophenyl phosphate (1 mg/ml, 10% diethanolamine, pH 9.8). ELISA absorbance values twice that of nontransgenic papaya were regarded as positive reactions. Glucoronidase expression (GUS) in leaf tissue was detected by the standard histochemical GUS assay (12). Double antibody sandwich ELISA (DAS ELISA) was carried out as previously described (13) for PRV CP detection in transgenic seedlings. The reaction was measured 60 min after substrate addition.

Western blot analysis. Total soluble protein from young leaf tissue of transgenic papaya (representative progenies from five different transgenic papaya trees of the 55-1 line from which the fruits and seeds were obtained) was homogenized (1:4, w/v) in extraction buffer (150 mM Tris-HCl pH 6.8, 10% sodium dodecyl sulfate [SDS], 25% 2-mercaptoethanol) (2). Twenty microliters of the extract were electrophoresed in a 12% SDS polyacrylamide gel and subsequently transferred to an Immobilon-P membrane (Millipore Corporation, Bedford, MA) (25). The membrane was then blocked with 2% bovine serum albumin (BSA) in PBS (0.14 M NaCl, 2 mM KH_2PO_4, 8 mM Na_2HPO_4, and 3 mM KCl) for 1 h at 4 C, washed with 0.05% Tween-20 and PBS for 30 min, and incubated with 2 μg/ml of anti-PRV IgG and 10% healthy leaf extract in 2% BSA in PBS overnight at 4 C. Following two 10-min washings with 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5 and 0.05% Tween-20, CP was detected by incubation in a protein A gold conjugate solution and silver enhancement (11).

Northern blot analysis. Total RNA was isolated from young leaves of transgenic seedlings as described previously (18) and electrophoresed in a denaturing formaldehyde 1.2% agarose gel (30 μg/lane) (22). The RNA gel was blotted onto a Gene Screen Plus nylon membrane following the manufacturer's manual (DuPont Co., Boston, MA) and probed with ^{32}P labeled *Hind III* fragment of the PRV HA 5-1 CP gene (5,6,7).

PRV isolates from different geographical regions. PRV isolates were collected from infected papaya in Mexico (isolates 12 and 17), the Bahamas, Florida (isolates F and G), Australia, Brazil, the People's Republic of China, Okinawa, Ecuador, Guam, Thailand, Jamaica, and Hawaii (isolates HA 5-1, HA, HA-Oahu, HA-Panaewa). It should be noted that PRV HA was originally collected from the island of Oahu, Hawaii, in 1977 and served as the virus source for nitrous acid mutation and derivation of the mild PRV HA 5-1 (28). HA-Oahu and HA-Panaewa were recently collected, in 1992. All isolates were maintained in the greenhouse on papaya and *C. metuliferus*. The serological relationship between PRV HA 5-1 and HA and the isolates from other regions was confirmed by DAS-ELISA (13) and SDS-immunodiffusion tests (9) with PRV HA 5-1 antisera and leaf extracts of *C. metuliferus* infected with the PRV isolates. All samples for both tests were collected 21 days after inoculation.

Inoculation of transgenic papaya with the severe PRV HA isolate. Five- to eight-wk-old seedlings (6–10 leaf stage, height 6–15 cm) were used for the inoculations with PRV HA. Transgenic seedlings were identified by NPTII-ELISA. Nontransgenic seedlings were included as controls. The seedlings were dusted with Carborundum and inoculated by one of the following three methods:

1) *One to two mechanical inoculations.* The three youngest fully expanded leaves of five sets of 10 transgenic seedlings were inoculated with leaf extract dilutions (1:1, 1:5, 1:10, 1:15, 1:20 in 0.01 M phosphate buffer pH 7.5) of *C. metuliferus* infected with PRV HA for 21 days. Nontransgenic papaya were similarly treated. Symptom development was monitored daily for 6 wk. Disease resistance was assessed by comparing the rate of symptom development and the severity of symptoms (vein clearing, mottling, leaf distortion) on transgenic and nontransgenic seedlings. Symptomless inoculated plants were then reinoculated and checked for virus infection after 3 wk by a virus recovery assay that involved inoculating nontransgenic *C. papaya*, *C. metuliferus*, and the local lesion host *C. quinoa* with leaf extracts from the symptomless new growth of the inoculated plants. The experiment was repeated.

2) *Multiple mechanical inoculations.* The new growth of a set of 10 transgenic seedlings was inoculated every 2–4 wk for 10 mo with a 1:20 dilution of PRV HA infected *C. metuliferus* leaf extract. Two nontransgenic plants of comparable age were similarly treated at each inoculation. All plants were maintained in the greenhouse up to 6 mo after the last inoculation. Disease resistance was assessed and virus infection checked as described above.

3) *Graft inoculation.* Ten transgenic seedlings were inoculated by approach grafting to nontransgenic seedlings inoculated with PRV HA. Nontransgenic seedlings were similarly grafted to PRV HA–infected nontransgenic seedlings as controls. The plants were maintained in the greenhouse up to 6 mo after the last inoculation. Disease resistance was assessed as described. Symptomless grafted plants were checked for virus infection by ELISA and by virus recovery assays described above.

Inoculation of transgenic papaya with PRV isolates from various geographical regions. Transgenic seedlings were mechanically inoculated with a 1:20 dilution of leaf extracts of *C. metuliferus* individually infected for 21 days with the 11 PRV isolates described above. Nontransgenic seedlings were similarly inoculated. At least three experiments with 10 seedlings were conducted. All inoculated plants were observed daily for 6 wk. Disease resistance was assessed by comparing the rate of symptom development and the severity of symptoms on the transgenic and nontransgenic seedlings. Symptomless inoculated plants were reinoculated and checked for virus infection by virus recovery assays as described above.

Cross protection assays with PRV isolates from various geographical regions. Of 35 Sunrise papaya seedlings (5–8 wk), 20 were inoculated on the three youngest fully expanded leaves with PRV HA 5-1 (28). Inoculum (1:10) was prepared from leaf extracts of *C. metuliferus* infected with PRV HA 5-1 for 21 days. Infection of the seedlings was confirmed after 21 days by ELISA using antisera to PRV HA 5-1 as described above. Ten HA 5-1–infected and 10 healthy papaya seedlings were then challenge inoculated with leaf extracts of *C. metuliferus* (1:20) infected with a severe PRV isolate. Separate assays were done with severe isolates from the Bahamas, Mexico, Brazil, Ecuador, Jamaica, China, Thailand, Florida, Australia, and Hawaii (two isolates, HA and HA-Panaewa). The remaining 10 HA 5-1–infected papaya and five healthy seedlings served as nonchallenged controls. Symptom expression was monitored for 6 wk. Disease development in the HA 5-1–infected and challenged papaya was assessed by comparison with symptom development in the seedlings inoculated only with a severe isolate. Challenged seedlings that failed to develop symptoms after 6 wk were reinoculated with the severe isolate and symptomless challenged plants were checked for virus infection after 3 wk by virus recovery assays as described above.

RESULTS

Analysis of transgenic papaya. Fruits of the R_0 transgenic Sunset line 55-1 produced large numbers of viable seeds following fertilization with pollen from nontransgenic Sunrise papaya. Since

the NPTII, CP, and GUS genes were linked on the plasmid pGA482GG/cpPRV-4 (7), it was expected that the transgenes would segregate 1:1. This was the case. Initial segregation tests (ELISA and GUS, R. Manshardt, *unpublished*) with R_1 seedlings showed that the CP gene segregated 1:1 also indicating a single gene locus insert in the 55-1 line. With the progenies of the crosses in Geneva, the NPTII test was used to screen the transgenic and nontransgenic papaya since the differences between the two were obvious within 10 min unlike the ELISA and X-Gluc tests (for the detection of the CP and GUS genes respectively). Fifty-two percent of 2,318 seedlings were NPTII positive (NPTII+). As with the R_0 plants previously tested, the 1.4-kb PRV-CP transcript was detected by Northern blot analysis of total RNA isolated from leaf extracts of the 55-1 progeny using a DNA probe of the CP gene (7). Interestingly, a larger band of 4.4 kb that was previously detected in R_0 55-1 plants was also detected in the progeny (data not shown).

ELISA tests measuring PRV CP showed that the levels of CP varied in a population of 698 NPTII$^+$ 55-1 progeny. Forty-three percent of the seedlings had ELISA absorbance values between 0.1–0.2, 32% between 0.05–0.1, and 25% between 0.2–1.1. One hundred nanograms of purified mild PRV HA 5-1 gave an absorbance reading of 0.19. The CP in the transgenic seedlings was also detected by Western blot analysis (Fig. 1). Transgenic seedlings showed a 28-kDa band that reacted with the CP antisera while purified PRV HA 5-1 and PRV HA 5-1–infected *C. metulifersus* showed a dominant band of 33 kDa and a minor band at 28 kDa. On the other hand, nontransgenic papaya infected with PRV HA 5-1 showed a dominant 28-kDa band.

Properties of PRV isolates from different geographical regions. Besides PRV HA, 11 severe PRV isolates from Mexico, the Bahamas, Florida, Australia, Brazil, the People's Republic of China, Okinawa, Ecuador, Guam, Thailand, Jamaica, and two isolates recently collected from Hawaii were used to challenge inoculate the progeny of transgenic 55-1 and papaya infected with the mild HA 5-1. These isolates were serologically indistinguishable from PRV HA 5-1 and PRV HA in SDS-immunodiffusion tests using antiserum to PRV HA 5-1 (Fig. 2). ELISA with monoclonal antibodies, however, gave strong but variable absorbance readings to the isolates. In four independent ELISA tests, the average absorbance readings ranged from about 0.5 for isolates from Jamaica, Guam, Thailand, to 1.8 for Brazil, Australia, PRV HA-Oahu, and PRV HA. The other isolates gave average readings ranging from 0.8–1.5. The ELISA absorbance readings for each isolate showed little variation over crude sap dilutions of 400–1,600. As expected, no reactions were observed in ELISA involving healthy papaya leaves or leaves infected with tobacco etch and pepper mottle potyviruses, or alfalfa mosaic from the alfalfa mosaic virus group.

The reactions of the severe PRV isolates were compared on Sunrise papaya, *C. metulifersus*, and the local lesion host *C. quinoa*. Symptoms on papaya included vein-clearing, mosaic patterns, distortion of the leaves, and stunting of the plant. Inoculated plants developed symptoms within 8–21 days depending on the size of the plant at the time of inoculation. Larger plants took a longer time to develop symptoms. Overall, the isolates showed differences in severity of symptoms and time for symptom development. The most severe isolates, such as PRV Thailand, generally induced symptoms within 10 days after inoculation while symptom development with less severe isolates, such as PRV Australia, occurred within 21 days. On the other hand, the severe isolates produced similar mosaic and leaf puckering on *C. metulifersus* and chlorotic or necrotic lesions on *C. quinoa* within 21–25 days. As previously described (28), PRV HA 5-1 produced mild symptoms on papaya and *C. metulifersus*, but no local lesions on *C. quinoa*.

Inoculation of transgenic papaya with PRV HA. Although the transgenic line 55-1 expresses the CP gene of PRV HA 5-1, this isolate was not used as the challenge virus because it produces only mild or no symptoms on papaya (28). Instead, transgenic seedlings were inoculated with the severe PRV HA, the isolate from which PRV HA 5-1 was derived (26,30). Transgenic seedlings were exposed to high disease pressure when challenge inoculated with PRV HA by one of three methods: one to two mechanical inoculations, multiple mechanical inoculations, or graft inoculation.

Forty-four transgenic seedlings were given a single inoculation with a 1:20 dilution of PRV HA–infected *C. metulifersus* leaf extract. The seedlings remained symptomless for the 6-wk duration of the experiment and up to the time they were discarded (3 mo). However, similarly treated nontransgenic seedlings developed typical PRV symptoms 13 days after inoculation. The infectivity of the inoculum used was tested on the local lesion host *C. quinoa*. More than 200 local lesions per half leaf were induced on *C. quinoa* (three leaves were inoculated). Other dilutions of 1:1, 1:5, 1:10, and 1:15 caused numerous lesions on *C. quinoa*, the exact number of which could not be accurately determined. When transgenic seedlings were inoculated with these dilutions (20 plants per dilution), they also remained symptomless. Thus, a 1:20 dilution was used for subsequent inoculations. The symptomless transgenic papaya were subjected to virus recovery assays to test for latent infection with PRV HA. All tests were negative.

Neither the level of CP in the transgenic seedlings nor the age of the seedlings influenced the resistance. For example, of 31

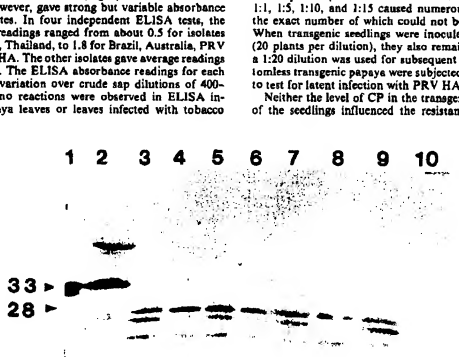

Fig. 1. Western blot analysis of coat protein in neomycin phosphotransferase II (NPTII) positive transgenic papaya. Total soluble protein of NPTII-positive transgenic papaya (lanes 4-9), *Cucumis metulifersus* infected with PRV HA 5-1 (lane 2), *Carica papaya* infected with PRV HA 5-1 (lane 3), and 200 ng of purified PRV HA 5-1 virions (lane 1), nontransgenic papaya (lane 10) were separated under denaturing conditions on a 12% polyacrylamide gel, blotted onto a nylon membrane, and probed with polyclonal antibodies to PRV HA 5-1. Antibody binding was visualized using protein A and silver staining.

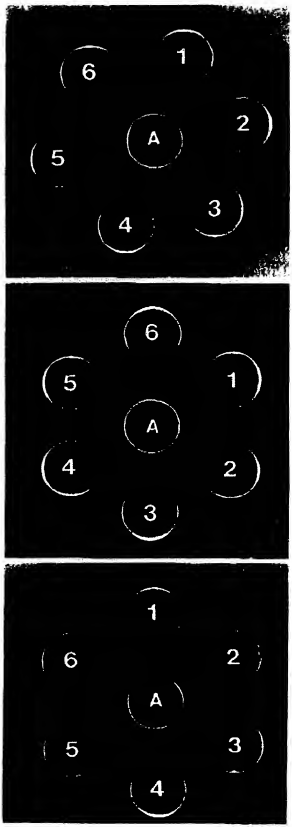

Fig. 2. SDS-immunodiffusion tests using antisera to PRV HA 5-1 (well A) against leaf extracts of *Cucumis metuliferus* infected with various severe PRV isolates in the peripheral wells. Leaf extracts from *C. metuliferus* infected with PRV HA 5-1 (wells 1, 3, and 5), PRV HA (well A2), PRV HA-Oahu (well A4), PRV Australia (well B2), PRV Thailand (well B4), and PRV HA-Panaewa (well C4), and healthy *C. metuliferus* leaf extract (well 6). Leaf extracts were prepared 21 days after inoculation.

transgenic seedlings between the ages of 5–8 wk that were inoculated with PRV HA, 26% had ELISA absorbance readings 0.05–0.1, 32% between 0.1–0.2, and 42% 0.2–1.1. All responded similarly to the inoculations and remained symptomless.

To evaluate the resistance of transgenic plants to multiple inoculations, 10 transgenic plants were inoculated 10 times over a period of 10 mo with a 1:20 inoculum preparation of PRV HA. All inoculated plants remained symptomless while similarly treated nontransgenic papaya developed symptoms within 24 days after the first inoculation. All virus recovery assays from transgenic papaya were negative.

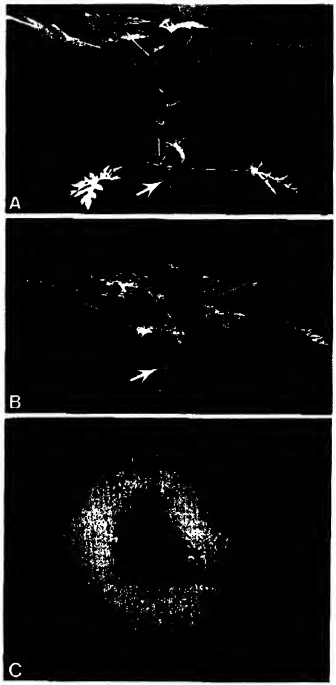

Fig. 3. Symptom development on graft-inoculated nontransgenic and transgenic papaya after 4 wk. A, PRV HA-infected nontransgenic papaya showing leaf distortion and plant stunting, grafted to transgenic papaya that did not develop symptoms; B, PRV HA-infected nontransgenic papaya grafted to nontransgenic papaya (both with leaf distortion and plant stunting); C, Graft union between transgenic (left) and nontransgenic papaya inoculated with PRV HA (right).

The resistance of the transgenic plants was further characterized by graft inoculation since our observations suggested that this was a more severe form of inoculation than mechanical inoculation. For example, Provvidenti and Gonsalves (19) recently showed that transgenic tomato expressing the CP gene of cucumber mosaic virus was resistant to mechanical inoculation but susceptible to graft inoculation. Twenty transgenic papaya seedlings were approach grafted to PRV HA–infected nontransgenic papaya seedlings. All of the graft-inoculated transgenic seedlings remained symptomless even after 3 mo (Fig. 3A) as well as a pair that were maintained in the greenhouse for a year. There was no apparent virus movement between the grafted seedlings as determined by the lack of symptom expression on the transgenic papaya and negative ELISA and virus recovery assays. On the other hand, all 10 nontransgenic seedlings grafted to nontransgenic seedlings infected with PRV HA developed symptoms within 24 days after the grafting (Fig. 3B). The conducting tissues of grafted plants were joined within 3 wk as determined by cross sections through the graft (Fig. 3C).

Reactions of transgenic papaya to severe PRV isolates from various geographical regions. The reactions of the transgenic seedlings to inoculations with PRV isolates from various geographical locations and the recently collected isolates from Hawaii are summarized in Table 1. The isolates recently collected from Hawaii failed to infect all but six of the 97 inoculated transgenic seedlings. These six infected seedlings were inoculated with PRV HA-Oahu and had detectable levels of CP (0.1–0.45 ELISA absorbance values) prior to inoculation. All seedlings inoculated with PRV HA-Panaewa remained symptomless as did the seedlings inoculated with the severe PRV HA (Fig. 4A).

PRV isolates from the Bahamas, Florida, and Mexico produced symptoms on 28–72% of the inoculated seedlings (Table 1). Conversely, the other seedlings remained symptomless even after reinoculation with the respective isolates. It was also observed that symptom development in the transgenic seedlings was delayed between 7 and 14 days and the symptoms were not as severe as those on nontransgenic seedlings. The level of CP expression in the inoculated plants did not correlate with the level of resistance

TABLE 1. Response of transgenic, nontransgenic, and mild strain-infected papaya to inoculations with PRV isolates from various geographical regions

	Plants (%) with symptoms after inoculation (days)													
	Transgenic					Nontransgenic				Mild strain-infected				
PRV isolates	n[a]	10	21	42	SE[b]	n	10	21	42	n	10	21	42	SE
Hawaii:														
-HA	31	0	0	0	NS[c]	43	0	100	100	28	0	0	0	NS
-HA-Oahu	65	0	6	6	S[d]	27	0	100	100	...[e]	...	...	...	...
-HA-Panaewa	32	0	0	0	NS	20	0	100	100	15	0	0	0	NS
Bahamas	45	0	24	29	A[f]	26	0	100	100	20	0	5	42	A
Mexico-17	41	4	29	44	A	24	63	100	100	20	0	0	20	A
Mexico-12	36	5	17	28	A	10	90	100	100	...	...	...	...	...
Florida-G	48	32	32	44	A	15	67	100	100	...	...	...	...	...
Florida-F	30	32	60	72	A	24	42	100	100	20	0	75	100	A
Australia	35	0	29	100	A	23	0	100	100	19	0	33	83	A
China	36	0	82	100	A	27	87	100	100	20	0	0	90	A
Jamaica	28	0	96	100	A	24	100	100	100	21	0	70	100	A
Guam	40	0	68	100	S	26	94	100	100	...	...	...	...	...
Brazil	29	0	94	100	S	20	100	100	100	20	0	60	100	S
Thailand	38	11	100	100	S	26	100	100	100	20	0	100	100	S
Ecuador	46	12	100	100	S	28	100	100	100	15	0	63	100	S
Okinawa	39	30	100	100	S	14	100	100	100	...	...	...	...	...

[a] Number of seedlings inoculated.
[b] Symptom expression.
[c] No symptom expression.
[d] Severe symptom expression.
[e] Not tested.
[f] Symptom attenuation.

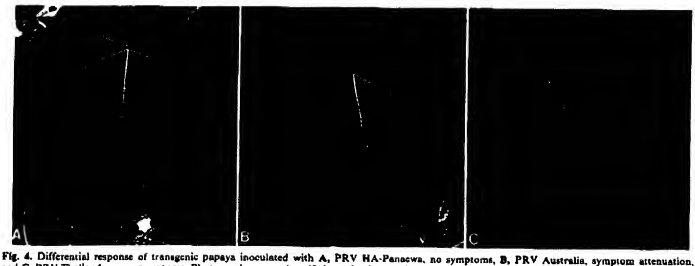

Fig. 4. Differential response of transgenic papaya inoculated with **A**, PRV HA-Panaewa, no symptoms, **B**, PRV Australia, symptom attenuation, and **C**, PRV Thailand, severe symptoms. Photographs were taken 42 days after inoculation.

observed in the transgenic seedlings. For example, of the 36 transgenic seedlings inoculated with PRV Mexico-12, the 28% that developed symptoms had ELISA absorbance readings between 0.068–0.186 prior to inoculation while the remaining seedlings that were symptomless had comparable readings between 0.067–0.226.

The PRV isolates from other regions induced systemic infection in all the transgenic seedlings and could be grouped according to the delay in symptom expression and the reduced virulence on transgenic seedlings (Table 1). Symptoms caused by the isolate from Australia, for example, were delayed up to 2 wk on transgenic seedlings and were less severe than those induced on nontransgenic papaya (Fig. 4B). Other isolates in this group were from Jamaica and China. However, symptoms caused by the isolate from Thailand were delayed by only 2–5 days on transgenic seedlings and were as severe as those induced on the nontransgenic seedlings (Fig. 4C) regardless of the CP accumulation in the seedlings. For example, of the 38 transgenic seedlings inoculated, 37% had ELISA absorbance readings 0.05–0.1, 8% between 0.1–0.2, and 55% 0.2–1.1, and all responded similarly to the inoculations with PRV Thailand. The isolates from Guam, Brazil, Ecuador, and Okinawa were also in this group.

Reactions of mild strain–infected papaya to PRV isolates from various geographical regions. Reactions of PRV HA 5-1–infected papaya seedlings challenge inoculated with two severe isolates from Hawaii and PRV isolates from other countries showed a trend similar to that of transgenic seedlings (Table 1). High levels of protection were observed against infection with PRV HA (Fig. 5A) and PRV HA-Panaewa. However, there were instances in which the noninoculated new growth of the protected seedlings devel-

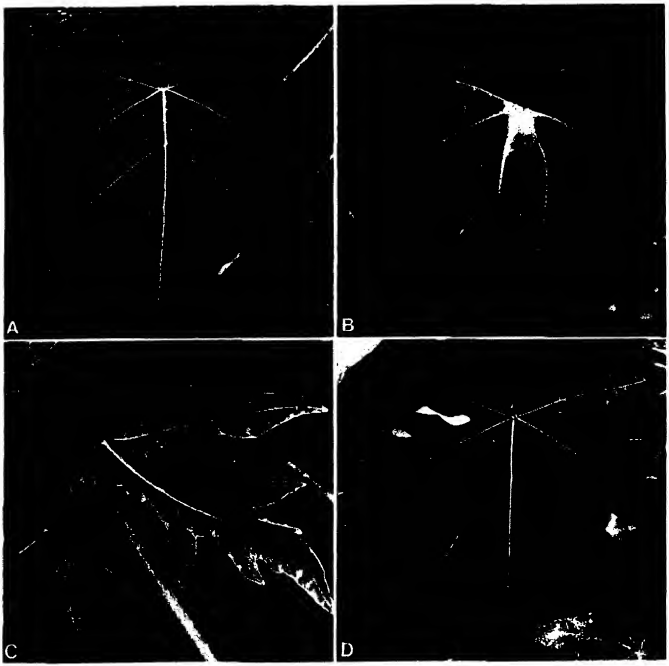

Fig. 5. Differential response of PRV HA 5-1–infected papaya challenged with the severe isolates A, PRV HA, no symptoms, B, PRV Jamaica, attenuated symptoms, C, PRV Thailand, severe symptoms. Papaya inoculated with PRV HA 5-1 and not challenged with a severe PRV isolate is shown in D. Photographs were taken 42 days after inoculation.

oped mild mosaic symptoms after inoculation with PRV HA and the subsequent growth was symptomless. Interestingly, tissue extracts from symptomless leaves of these papaya induced severe symptoms on nonprotected papaya and *C. metuliferus* and local lesions on *C. quinoa*.

Lesser levels of protection were observed against isolates collected from outside of Hawaii. Challenge inoculations involving isolates from the Bahamas, Mexico, and Australia did not result in disease development in all seedlings (17–80%). There was also a delay in symptom expression of 1–3 wk, and symptoms were attenuated. Virus recovery tests from symptomless seedlings following challenge inoculation were positive for the severe strain. Other isolates induced disease on all the challenged seedlings. However, there was a delay in symptom development and symptom attenuation with isolates from Florida and Jamaica (Fig. 5B) but a shorter delay in symptom development and no symptom attenuation with isolates from Ecuador, Okinawa, and Thailand (Fig. 5C).

DISCUSSION

We have shown that progenies of a transgenic papaya line with the CP gene of a mild strain of PRV from Hawaii show exxremely high levels of resistance to three PRV isolates from Hawaii but little or no resistance to isolates from other geographical regions. Prior inoculation of papaya with the mild PRV HA 5-1 strain gave similar levels of protection against severe isolates from Hawaii but conferred only partial protection against other isolates. This is the first report that compares CPMP and classical cross protection in a crop where the resistant gene for CPMP is from the mild strain of the virus that was used in the cross protection experiments.

The PRV CP that accumulated in the transgenic papaya was 28 kDa which is somewhat smaller than the expected PRV CP (34 kDa) based on the coding capacity of the chimeric construction that fused 16 amino acids of the cucumber mosaic virus (CMV) CP gene to the PRV CP (13). It is likely that the chimeric CMV-PRV CP in the transgenic papaya is being cleaved by a plant protease in papaya. A 28-kDa protein along with the apparent native CP was also observed in nontransgenic papaya and *C. metuliferus* infected with PRV HA 5-1. Lower molecular weight forms of CP in purified virus preparations and crude plant sap have been reported for potyviruses (10).

The severe PRV isolates used in this study were serologically indistinguishable from the mild PRV HA 5-1 and PRV HA and their biological properties on various PRV hosts were also similar. However, the transgenic papaya showed complete resistance only against PRV isolates from Hawaii. That is, inoculation with high concentrations of the virus, multiple mechanical inoculations, or graft inoculation failed to break the resistance of the transgenic papaya. Virus recovery assays from these plants suggest that virus replication and movement were impaired in transgenic papaya. Transgenic papaya also showed high levels of resistance against PRV HA-Panaewa, which was recently collected from Hawaii. On the other hand, 9% of the transgenic papaya inoculated with another recently collected isolate from Hawaii, PRV HA-Oahu, developed systemic infections. It is possible that the PRV HA-Oahu is a mixture of strains some of which can break down the resistance of the line 55-1.

By contrast, differential resistance was observed when transgenic papaya were inoculated with PRV isolates from other regions. The plants showed a variety of reactions including complete resistance, delay and attenuation of symptoms, or delay in symptom development but no attenuation. This range is typical of CPMP against potyviruses and degrees of heterologous protection have also been reported (4,17,24). Interestingly, heterologous CPMP has been reported with PRV CP HA 5-1 gene in tobacco (13). Transgenic tobacco showed a significant delay in symptom development and symptom attenuation when inoculated with tobacco etch, potato virus Y, and pepper mottle potyviruses. This occurrence of CPMP providing complete resistance to isolates from one region but differential resistance to isolates from other geographical regions has not been previously reported. It is possible that the CP gene used for the transformations is responsible for the limited spectrum of resistance in the 55-1 transgenic papaya. It has been shown that the CP gene of the white leaf strain of CMV offers a broader spectrum of resistance against various CMV strains than the CP gene of the C strain of CMV (16).

Classical cross protection with the mild PRV HA 5-1 strain also provided high levels of protection against PRV HA and PRV HA-Panaewa, but lesser levels of protection were observed against isolates from other countries. These results are similar to field data from Hawaii where HA 5-1 provides economical protection to papaya and from Taiwan (27) where HA 5-1 is much less effective. Protection against PRV HA in HA 5-1–infected papaya was not as complete as that in the transgenic papaya. There were instances of mild symptom development followed by recovery in the cross-protected papaya. Furthermore, the virus recovery assays suggest that replication and movement of the severe strain were not as completely impaired in the symptomless cross-protected papaya as in the symptomless transgenic papaya.

In conclusion, this study has shown that CPMP in papaya, like classical cross protection, is highly effective against PRV isolates from Hawaii. Transgenic papaya, unlike the cross-protected papaya, do not appear to support virus replication or movement when challenged with the PRV HA isolate and should therefore be useful for the control of PRV disease in Hawaii. In fact, R_0 55-1 plants have not become diseased after 24 mo in the field in Hawaii (R. Manshardt, *unpublished*). On the other hand, both CPMP with PRV HA 5-1 CP gene and cross protection with PRV HA 5-1 gave a range in effectiveness against PRV isolates from other geographical regions. Investigations with the CP genes of other PRV isolates are under way in order to identify CP sequences involved in CPMP and possibly broaden the spectrum of resistance to PRV.

LITERATURE CITED

1. Beachy, R. 1993. Virus resistance through expression of coat protein genes. Pages 89-104 in: Biotechnology in Plant Disease Control. Wiley-Liss, Inc., New York.
2. Brault, V., Candresse, T., le Gall, O., Delbos, R., Lannesu, M., and Dunez, J. 1993. Genetically engineered resistance against grapevine chrome mosaic nepovirus. Plant Mol. Biol. 21:89-97.
3. Cabanes-Bastos, E., Day, A. G., and Lichtenstein, C. P. 1989. A sensitive and simple assay for neomycin phosphotransferase II activity in transgenic tissue. Gene 77:69-176.
4. Dinant, S., Blaise, F., Kusiak, C., Astier-Manifacier, S., and Albouy, J. 1993. Heterologous resistance to potato virus Y in transgenic tobacco plants expressing the coat protein gene of lettuce mosaic potyvirus. Phytopathology 83:818-824.
5. Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.
6. Fitch, M. M. M., Manshardt, R. M., Gonsalves, D., Slightom, J. L., and Sanford, J. C. 1990. Stable transformation of papaya via microprojectile bombardment. Plant Cell Rep. 9:189-194.
7. Fitch, M. M. M., Manshardt, R. M., Gonsalves, D., Slightom, J. L., and Sanford, J. C. 1992. Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus. Bio/Technology 10:1466-1472.
8. Gonsalves, D., and Garnsey, S. M. 1989. Cross-protection techniques for control of plant virus diseases in the tropics. Plant Dis. 73:592-596.
9. Gonsalves, D., and Ishii, M. 1980. Purification and serology of papaya ringspot virus. Phytopathology 70:1028-1032.
10. Hiebert, E., and McDonald, J. 1976. Capsid protein heterogeneity in turnip mosaic virus. Virology 70:144-150.
11. Hu, J. S., Gonsalves, D., Boscia, D., and Namba, S. 1990. Use of monoclonal antibodies to characterize grapevine leafroll associated closteroviruses. Phytopathology 80:920-925.
12. Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5:387-405.
13. Ling, K., Namba, S., Gonsalves, C., Slightom, J. L., and Gonsalves, D. 1991. Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. Bio/Technology 9:752-758.
14. Manshardt, R. M. 1992. Papaya. Pages 489-511 in: Biotechnology

of Perennial Fruit Crops. F. A. Hammerschlag and R. E. Litz, eds. CAB International. Kew, UK.
15. Mau, R. F. L., Gonsalves, D., and Renato, B. 1989. Use of cross-protection to control papaya ringspot virus at Waianae. Pages 77-84 in: Proceedings of the Hawaii Papaya Industry Association Twenty-fifth Annual Conference. C. L. Chia, ed. Hilo, Hawaii.
16. Namba, S. Ling, K., Gonsalves, C., Slightom, J. L., and Gonsalves, D. 1991. Expression of the gene encoding the coat protein of cucumber mosaic virus (CMV) strain WL appears to provide protection to tobacco plants against infection by several CMV strains. Gene 107:181-188.
17. Namba, S., Ling K., Gonsalves, C., Slightom, J. L., and Gonsalves, D. 1992. Protection of transgenic plants expressing the coat protein gene of watermelon mosaic virus II or zucchini yellow mosaic virus against six potyviruses. Phytopathology 82:940-946.
18. Napoli, C., Lemieux, C., and Jorgensen, R. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 2:279-289.
19. Provvidenti, R. and Gonsalves, D. 1994. Inheritance of resistance to cucumber mosaic virus in a transgenic tomato line with the coat protein gene of the white leaf strain. J. Hered. (In press.)
20. Purcifull, D. E., Edwardson, J. R., Hiebert, E., and Gonsalves, D. 1984. Papaya ringspot virus. CMI/AAB Descriptions of Plant Viruses, no. 84, revised.
21. Quemada, H., L'Hostis, B., Gonsalves, D., Reardon, I. M., Heinrickson, R., Hiebert, E. L., Sisu, I. C., and Slightom, J. L. 1991. The nucleotide sequences of the 3' terminal regions of papaya ringspot virus strains W and P. J. Gen. Virol. 70:203-210.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1991. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring, NY.
23. Sanford, J., and Johnston, S. 1985. The concept of parasite derived-resistance genes from the parasite's own genome. J. Theor. Biol. 113:395-405.
24. Stark, D., and Beachy, R. 1989. Protection against potyvirus infection in transgenic plants: Evidence of broad spectrum resistance. Bio/Technology 7:1257-1262.
25. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76:4350-4354.
26. Wang, C. H., and Yeh, S.-D. 1992. Nucleotide sequence comparison of the 3' terminal regions of severe, mild, and non-papaya infecting strains of papaya ringspot virus. Arch. Virol. 127:345-354.
27. Wang, H.-L., Yeh, S.-D., Chiu, R.-J., and Gonsalves, D. 1987. Effectiveness of cross protection by mild mutants of papaya ringspot virus for control of ringspot disease of papaya in Taiwan. Plant Dis. 71:491-497.
28. Yeh, S.-D., and Gonsalves, D. 1984. Evaluation of induced mutants of papaya ringspot virus for control by cross protection. Phytopathology 74:1086-1091.
29. Yeh, S.-D., and Gonsalves, D. 1994. Practices and Perspective of Control of Papaya Ringspot virus by Cross Protection. In: Advances in Disease Vector Research. (In press.)
30. Yeh, S.-D., Jan, F., Chiang, C., Doong, T., Chen, M., Chung, P., and Bau, H. 1992. Complete nucleotide sequence and genetic organization of papaya ringspot virus. J. Gen. Virol. 73:2531-2541.

Techniques

Differentiation of Two Closely Related Furoviruses Using the Polymerase Chain Reaction

C. M. Rush, R. French, and G. B. Heidel

First and third authors: Texas Agricultural Experiment Station, P.O. Drawer 10, Bushland, TX 79012; and second author: USDA-ARS, 406 Plant Science, Lincoln, NE 68583.
Accepted for publication 30 August 1994.

ABSTRACT

Rush, C. M., French, R., and Heidel, G. B. 1994. Differentiation of two closely related furoviruses using the polymerase chain reaction. Phytopathology 84:1366-1369.

Oligonucleotide primers based on published sequence data for beet necrotic yellow vein virus (BNYVV) were synthesized for use in the reverse transcriptase polymerase chain reaction (RT-PCR) to differentiate beet soilborne mosaic virus (BSBMV) from BNYVV. Primers designed for the 3' end of BNYVV RNA 1 were effective in PCR amplification of a product of the predicted size, approximately 1,056 bp, from extracts of plants infected by BNYVV. The same primer pair also directed the amplification of a PCR product of approximately 1,000 bp from extracts of plants infected by BSBMV. If extracts from plants infected with BNYVV were mixed with those from plants infected with BSBMV, the primer pair allowed the amplification of only BNYVV. In addition to the slight size difference, the BSBMV product could be distinguished from the BNYVV product by digestion with *Tha*I, which cleaved the BSBMV product but not the BNYVV product. The BSBMV RT-PCR product was partially sequenced, and primers specific for BSBMV were synthesized. The primers directed the amplification of a PCR product of the predicted size, approximately 691 bp, only with extracts from plants infected by BSBMV. Only one PCR product of the size expected for BSBMV was produced from extracts containing both BSBMV and BNYVV. The BSBMV PCR product obtained with the BSBMV-specific primers could be digested by *Tha*I. PCR products of similar size were amplified using the BSBMV primers and extracts of several isolates of BSBMV differing in geographic origin and symptom phenotype.

Rhizomania, caused by beet necrotic yellow vein virus (BNYVV), was first reported in the United States in California in 1984 (3). It was next identified in Texas in 1987 (2). The disease was thought to be restricted to these two states, but during 1992–1993, rhizomania was found in Colorado, Idaho, Nebraska, and Wyoming (4,5). Because of the importance of the sugar beet industry in these states, intensive programs to determine the distribution of BNYVV were established. However, the presence of another soilborne virus infecting sugar beet, initially designated as Tx7, complicated and confused detection and identification of BNYVV (10).